This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

0013-7227/95/$03.00/0
Endocrinology
Copyright © 1995 by The Endocrine Society
Vol. 136, No. 6
Printed in U.S.A.

Interleukin 4, Interferon-γ, and Prostaglandin E Impact the Osteoclastic Cell-Forming Potential of Murine Bone Marrow Macrophages*

DAVID L. LACEY, JEANNE M. ERDMANN, STEVEN L. TEITELBAUM, HONG-LIN TAN, JUNICHI OHARA, AND ATSUSHI SHIOI†

Department of Pathology, Jewish Hospital at Washington University (D.L.L., J.M.E., S.L.T., H.-L.T., A.S.), St. Louis, Missouri 63110; and the Division of Gastroenterology, University of Colorado School of Medicine (J.O.), Denver, Colorado 80262

ABSTRACT

Interleukin 4 (IL-4) is an immune cytokine that inhibits bone resorption in mice and suppresses osteoclastic cell formation *in vitro* through an undefined mechanism. In this report, we have established the cellular identity of the IL-4 target cell using a variety of bone marrow/stromal cell coculture methods. Initially, we found that the majority of IL-4's inhibition of osteoclastic cell formation was due to its effect on bone marrow cells, not stromal cells. Consequently, bone marrow macrophages were used as osteoclastic cell progenitors after they had been transiently exposed to IL-4 (48 h), before the addition of stromal cells, 1,25-dihydroxyvitamin D_3, and dexamethasone. In this circumstance, IL-4 impaired subsequent osteoclastic cell formation, suggesting that the macrophage may be potentially targeted by many factors known to influence osteoclast formation. Consequently, we discovered that interferon-γ (IFNγ), prostaglandin E (PGE), and cell-permeant cAMP analogs also impacted osteoclastic cell formation when used to selectively treat bone marrow macrophages. IFNγ suppressed osteoclastic cell formation, whereas PGE and cAMP analog treatment led to the formation of significantly enlarged osteoclastic cells. Importantly, PGE antagonized the inhibitory effects of both IL-4 and IFNγ on the osteoclastic cell-forming potential of bone marrow macrophages. Collectively, these findings establish bone marrow macrophages as osteoclastic cell precursors with the degree of their commitment to the osteoclast pathway sensitive to the effects of soluble mediators, including IL-4, IFNγ, and PGE. (*Endocrinology* **136:** 2367–2376, 1995)

INTERLEUKIN 4 (IL-4) is a 20-kilodalton immunoregulatory glycoprotein that is produced and secreted by activated T lymphocytes and mast cells. This cytokine functions as a growth and/or differentiation factor for a wide variety of cells of hematopoietic lineage (1–7). In addition to these effects, IL-4 has been shown to influence skeletal metabolism (8). In this study, IL-4 inhibited the *in vitro* bone resorption induced by a wide variety of agents [PTH, PTH-related peptide (PTHrP), 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], IL-1α, IL-1β, and prostaglandin E_2 (PGE_2)], which in excess have been implicated in both osteoporosis and hypercalcemia of malignancy (9). This inhibitory effect of IL-4 on bone resorption was neutralized by the addition of a monoclonal anti-IL-4 antibody. These observations have been extended to the intact animal, as IL-4 has been shown to antagonize bone resorption stimulated in mice by infusions of PTHrP or by PTHrP- and IL-1α-expressing tumors (10).

Although these observations established IL-4 as a potent antiosteolytic factor, the mechanism(s) underlying its effects remained poorly characterized. Because IL-4 inhibited the resorption-inducing effects of such a diverse group of osteolytic substances, we reasoned that this cytokine impacted essential resorptive processes. In support of this hypothesis, we documented that IL-4 significantly antagonizes osteoclast generation *in vitro* (11) in an *in vitro* hematopoietic model of osteoclastic cell formation using bone marrow cell/stromal cell cocultures. These findings were confirmed by others, who showed that IL-4 inhibits osteoclastic cell generation in whole bone marrow and stromal cell/spleen cell cocultures (12, 13).

Although the ability of IL-4 to impair osteoclastic cell generation was a novel observation, the processes and cells impacted by this cytokine that disrupt osteoclastic cell formation remain unclear. Because IL-4 has profound complex effects on the proliferation and maturation of a variety of hematopoietic cells (5, 7) that may be mediated by the effects of lymphokine on stromal cells (6), the principal objective of this study was to determine the IL-4 cellular target within the myriad of cells that comprise our coculture model. Furthermore, we reasoned that the identification of the IL-4 target cell must precede further attempts to characterize the mechanism of IL-4's inhibitory effects on *in vitro* osteoclastic cell formation. Once we had accomplished this task, we directed interferon-γ (IFNγ) and PGE, agents with previously described effects on *in vitro* bone resorption and osteoclastic cell formation (13–17), against the putative IL-4-sensitive cell to determine whether these agents also targeted the same cell. Finally, we present an updated model of *in vitro* osteoclast

Received December 12, 1994.
Address all correspondence and requests for reprints to: David L. Lacey, M.D., Department of Anatomic Pathology, Amgen, Inc., Mail Stop 15-2-A-250, 1840 DeHavilland, Thousand Oaks, California 91320-1789.
* This work was supported in part by grants from the NIH (AR-42356, DE-05413, and AR32788) and the Shriner's Hospital for Crippled Children (St. Louis Unit).
† Current address: Second Department of Internal Medicine, Osaka City University Medical School, 1-5-7 Asahi-machi, Abeno-ku, Osaka 545, Japan.

formation, originally outlined by Suda *et al.* (18), that incorporates our findings.

Materials and Methods

Mice and cell lines

C3H/HEN mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). ST2 cells (stromal cell line) were obtained from RIKEN Cell Bank (Tsukuba, Japan), maintained in α-Minimum Essential Medium supplemented with 10% heat-inactivated fetal calf serum (α10-MEM), passaged weekly after trypsinization, and used up to passage 10.

Reagents

Recombinant IL-4 was expressed and purified from baculovirus-transfected SF-9 (*Spodoptera frugiperda*) cell culture supernatants as previously described (19, 20) and was stored at −80 C. The biological potency of IL-4 was determined using the HT-2 (murine T cell line) proliferation assay (21), with the amount of material stimulating half-maximal proliferation equal to 1 U. Macrophage colony-stimulating factor (M-CSF) was purified from L929 cell-conditioned medium by a modification (22) of the method of Stanley and Heard (23). Murine IFNγ was obtained from Genzyme (Cambridge, MA). Pronase was purchased from Boehringer Mannheim (Indianapolis, IN). 1,25-$(OH)_2D_3$ was kindly provided by Milan Uskokovic (Hoffman LaRoche, Nutley, NJ). Unless otherwise stated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Bone marrow cell preparation

Bone marrow cells were prepared by a modification (11) of a previously described method (22, 24). Note that this method involves an initial overnight incubation to deplete the bone marrow cells of adherent cells, and this step includes the addition of M-CSF (500 U/ml).

Stromal cell (ST2 cells or calvarial osteoblastic cells)/bone marrow cocultures

Cocultures of bone marrow cells and ST2 cells were performed in several different ways (see Fig. 1), all of which resulted in the formation of osteoclastic cells. In the first method (Fig. 1A), termed the continuous coculture method, ST2 cells (1×10^5 cells/ml) were cultured for 24 h in α10-MEM in 96-well (0.2 ml) or 48-well (0.5 ml) plates. At this time, the ST2 culture supernatants were discarded, and the previously prepared nonadherent bone marrow cells (1×10^6 cells/ml) in α10-MEM containing 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M dexamethasone were added to the ST2 cell cultures (0.2 ml, 96-well plate; 0.5 ml, 48-well plate). The cultures were maintained for 10 days, with medium changed twice weekly.

In the second method, termed the nonadherent cell method (Fig. 1B), bone marrow and stromal cells were cultured for 4 days before their combination to allow for a pretreatment period. To prevent their adherence during this time, nonadherent bone marrow cells were cultured in Teflon beakers in α10-MEM supplemented with 1000 U/ml M-CSF. ST2 cells were cultured in α10-MEM in T-25 flasks. After a 4-day incubation, ST2 cells were trypsinized and suspended in α10-MEM. Then, ST2 cells and bone marrow cells were combined and cultured in α10-MEM containing 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M dexamethasone for 10 additional days.

In the third method, termed the adherent cell method (Fig. 1C), the nonadherent bone marrow cells, isolated after overnight incubation in M-CSF, were grown on tissue culture plates in the presence of 1000 U/ml M-CSF. After 3 additional days, nonadherent cells were rinsed away, and the remaining adherent cells (termed day 4 cells) were exposed to various conditions in the presence of M-CSF (1000 U/ml) for 48 h. After these treatments, adherent cells were again rinsed, and ST2 cells were added (1×10^5 cells/ml; 0.5 ml; 48-well plate well) in 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M dexamethasone containing α10-MEM. The cultures were maintained for an additional 8 days, with medium changed once.

Bone resorption assay (pit assay)

Thin disc-shaped wafers (7×1 mm) of cortical bone were prepared from transverse slices bovine femurs (diaphysis) with a low speed diamond saw (Buehler, Evanston, IL) and stored in absolute ethanol at 4 C. Before their use, the slices were washed with sterile PBS and placed in 48-well plate wells. Day 1 nonadherent bone marrow cells (5×10^5 cells/well) were cultured on the bone slices in 0.5 ml α10-MEM containing 1000 U/ml M-CSF for 3 days (protocol similar to those in Fig. 1C). Nonadherent cells were then rinsed from the wells, and various treatments were added. After 2 additional days, these treatments were rinsed away, and ST2 cells (5×10^4 cells/well), 10^{-8} M 1,25-$(OH)_2D_3$, and 10^{-7} M dexamethasone were added. After an additional 8 days (medium changed every 4 days), the bone pits (resorption lacunae) were visualized using a variation of a previously described method (25). The slices were exposed to 0.1 M sodium hydroxide for 15 min. The disc surfaces were vigorously rinsed with this solution, using a pipette to remove the cell layer from the bone. The slices were then rinsed in 1% (wt/vol) sodium borate and stained with toluidine blue solution [1% (wt/vol) in the borate solution] for 10 min. The slices were briefly rinsed in dye-free borate solution, air-dried, and then mounted on glass slides with Acrytol mounting medium (Surgipath Medical Industries, Richmond, IL). Pits appeared as darkly staining, clearly marginated areas using light microscopy.

Enzyme chemistry: tartrate-resistant acid phosphatase (TRAP) staining

The presence of generated osteoclastic cells was evaluated by cytochemical detection of TRAP using a commercial kit. A cell that was TRAP

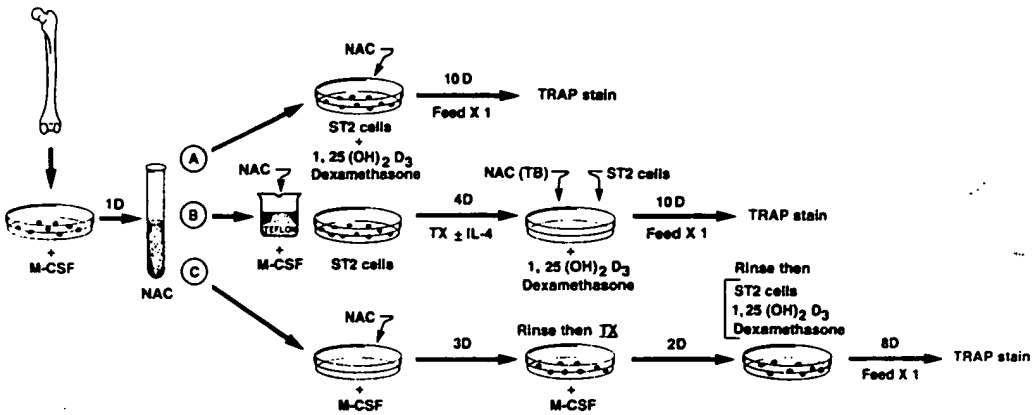

FIG. 1. Cell culture methods used for osteoclastic cell development. A, Continuous coculture method. B, Nonadherent cell method. C, Adherent cell method. D, Day; NAC, nonadherent cells; TB, Teflon beaker.

positive and contained three or more nuclei (TRAP-positive MNC) was considered an osteoclastic cell.

Image analysis

Osteoclastic cells on plastic dishes and toluidine blue-stained bone pits were viewed with a Nikon Diaphot (Nikon Corp., Melville, NY) or Labophot microscope (Nikon), respectively, equipped with a Sony XC77 camera (Sony Corp., Tokyo, Japan). The camera was connected to a digitizing scientific frame grabbing card (model LG-3, Scion Corp., Frederick, MD) located in a Macintosh II computer. The osteoclastic cell and pit images were traced with the assistance of a digital tablet (ArtZ, WACOM, Vancouver, WA) and image analysis software (NIH IMAGE, version 1.47, public domain). To obtain measurements in units of measure, the system was calibrated using a slide with an inscribed line of known length.

[^{125}I]IL-4 binding assay and IL-4 receptor Northern blots

[^{125}I]IL-4 binding assays and Northern blots screened for the presence of IL-4 receptor transcripts were done as previously described (26).

Statistics

In certain experiments, the data were statistically assessed using analysis of variance. The differences between groups were tested for significance using Fisher's protected least significant difference test, with the cut-off level set at 5%. The Kolmogorov-Smirnov test was used for the pit area data due to the apparent skewness of the data. These analyses were performed with the assistance of a computer program (Statview 4.0, Abacus Concepts, Berkeley, CA).

Results

In an earlier account (11), we demonstrated that IL-4 inhibited osteoclastic cell formation using the continuous bone marrow cell/ST2 cell coculture system (Fig. 1A) over a cytokine range that also stimulated T cell proliferation. To determine whether this IL-4 effect was time of addition dependent, IL-4 was added at the time of culture initiation or on days 4 and 7 thereafter, and osteoclastic cell number was determined on day 10. As shown in Fig. 2, IL-4 inhibited osteoclastic cell formation only when it was added at the initiation of coculture (day 1). The addition of IL-4, when delayed to either day 4 or 7 of coculture, failed to impair osteoclastic cell formation. These results suggested that the principal effects of IL-4 occurred during the first 4 days of the coculture period through its effects on one of the many cells present in this complex culture system.

To further delineate the cell population impacted by IL-4, we designed a cross-over experiment (see Fig. 1B), the aim of which was to determine whether the bone marrow cells or ST2 cells were the IL-4 cellular targets. In these experiments, nonadherent bone marrow cells were cultured for 4 days in medium containing 1000 U/ml M-CSF with and without IL-4 (50 U/ml) in Teflon beakers, to which macrophages poorly adhere. In parallel with these bone marrow cultures, ST2 cells were cultured in medium with and without IL-4 (50 U/ml) for 4 days. The ST2 and bone marrow cells were then combined and cultured for 10 days in the presence or absence of IL-4 (50 U/ml). As summarized in Table 1, continuous IL-4 treatment (IL-4 in bone marrow cell, stromal cell, and their subsequent cocultures) led to a 70% inhibition of osteoclastic cell number compared to the control value. Importantly,

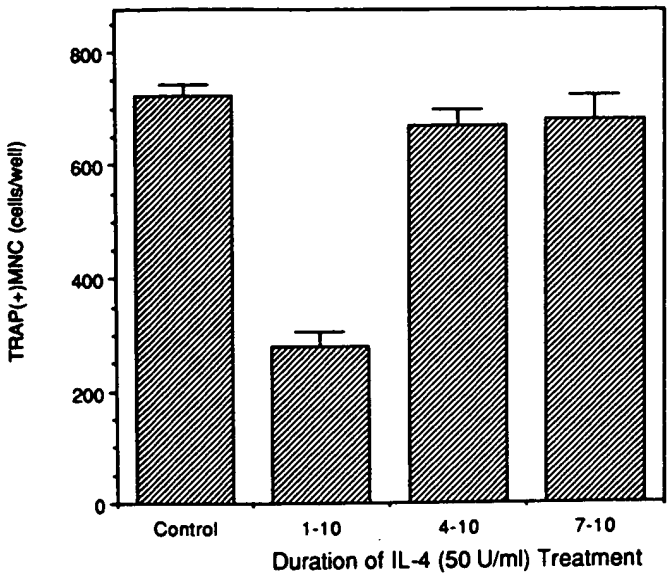

FIG. 2. IL-4's inhibitory effect on osteoclastic cell formation requires early exposure. ST2/nonadherent bone marrow cell cultures were established as described (Fig. 1A), and IL-4 (50 U/ml) was added at culture initiation or on days 4 and 7 thereafter. Osteoclastic cells were enumerated as described (see *Materials and Methods*). The results represent the mean (n = 4) ± SD number of osteoclastic cells per 96-well plate well. The 1–10 group is statistically different from all others ($P < 0.0001$).

TABLE 1. Effects of IL-4 on osteoclastic cell formation is largely mediated by cells within the bone marrow population

First culture[a]		Coculture[b]	TRAP-positive MNC (cells/well)[c]	% Inhibition[d]
BM cells	ST2 cells			
–[e]	–	–	693.2 ± 52.9	
–	–	+	504.0 ± 27.2	27.3
+	–	–	318.3 ± 34.7	54.1
+	–	+	249.5 ± 35.6	64.0
–	+	–	691.0 ± 23.8	0.3
–	+	+	483.3 ± 34.3	30.3
+	+	–	346.3 ± 53.4	50.1
+	+	+	210.3 ± 22.0	69.7

[a] Nonadherent bone marrow (BM cells) and ST2 cells were cultured separately for 4 days, as described in *Materials and Methods*.

[b] The BM cells (5×10^5 cells) and ST2 cells (5×10^4 cells) were then combined and cultured for 10 days in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M dexamethasone.

[c] Osteoclastic cells were identified as TRAP-positive cells containing three or more nuclei. The results are reported as the mean (n = 4) ± SD.

[d] This value is derived as follows: $(693.2 - x)/693.2 \times 100$. Three-way analysis of variance established that IL-4 treatment of BM cells ($P < 0.0001$) or the presence of IL-4 in cocultures ($P < 0.0001$) resulted in significant differences. IL-4 treatment of ST2 cells alone did not lead to significant effects.

[e] The + and – indicate that the culture was treated with either IL-4 (50 U/ml) or medium alone, respectively.

most of this inhibition (~77%) could be explained by the effect of IL-4 on bone marrow cells, because IL-4 bone marrow cell pretreatment alone led to a 54% reduction of osteoclastic cell number. Pretreatment of stromal cells with IL-4 essentially failed to impair osteoclastic cell number. These results suggested that the impact of IL-4 on a cell within the bone marrow cell compartment resulted in reduced oste-

oclastic cell formation in this system. The possibility that IL-4's apparent selective action on bone marrow cells was due to the lack of IL-4 receptor expression by ST2 or osteoclastic cell-containing cultures was excluded by experiments demonstrating that both of these cultures contained IL-4 receptor messenger RNA (mRNA; Northern blots) and cell surface IL-4 receptors ([^{125}I]IL-4 binding; data not shown).

The objective of the next series of experiments was to further define the IL-4 cellular target in the bone marrow cell population. Based on the observations that 1) tissue macrophages functioned as osteoclastic cell precursors (27); 2) M-CSF-deficient animals failed to produce osteoclasts (28, 29); and 3) we inhibited osteoclastic cell formation by selectively treating bone marrow cells raised with M-CSF and IL-4 (Table 1), we reasoned that the IL-4 cellular target was a M-CSF-responsive cell in the marrow compartment. To test whether bone marrow macrophages *per se* could function as osteoclastic cell progenitors, we cultivated the day 1 nonadherent bone marrow cells in M-CSF-containing medium for 3 days and then rinsed away the nonadherent cells. This results in the isolation of a M-CSF-dependent adherent cell population consisting principally of macrophages (30). When ST2 cells, 1,25-$(OH)_2D_3$ (10 nM), and dexamethasone (100 nM) were added, multinucleated TRAP-positive cells with features identical to those generated in the previous experiments developed over 8 additional days (data not shown). These findings indicated that bone marrow macrophages possessed the capacity to differentiate into osteoclastic cells given the appropriate conditions and, therefore, may be selectively targeted by agents known to alter *in vitro* osteoclastic cell formation, such as IL-4.

To test this hypothesis, day 4 bone marrow macrophages were exposed to various concentrations of IL-4 after the nonadherent cells had been rinsed from the wells. After an additional 48 h, the IL-4 treatment medium was removed, and ST2 cells were added together with 1,25-$(OH)_2D_3$ (10 nM) and dexamethasone (100 nM). Osteoclastic cell number was determined 8 days later. As shown in Fig. 3, IL-4 dose dependently inhibited osteoclastic cell development, with maximal inhibition (~75%) at 200 U/ml IL-4 and the 50% inhibitory dose (ID_{50}) of IL-4 being approximately 22 U/ml. The IL-4 ID_{50} in this circumstance was quite similar to the value we observed in experiments in which we had tested IL-4 in the continuous coculture model (IL-4 ID_{50} = 10 U/ml) (11).

These findings suggested that bone marrow macrophages were targeted by IL-4 and that this lymphokine altered (in this case impaired) the cells' osteoclastic cell-forming potential. To determine whether the osteoclastic cell-forming potential of the bone marrow macrophage at this stage of its *in vitro* differentiation was selectively impacted by IL-4, we tested other agents that had been previously shown to either inhibit (IFNγ) (13) or stimulate (PGE_1, PGE_2, and cAMP) (31) osteoclastic cell formation *in vitro*. In contrast to the previous osteoclastic cell development models that used unfractionated whole bone marrow cultures or stromal cell/spleen cell cocultures where these factors have been examined, we directed these agents directly against bone marrow macrophages transiently (48 h) before the addition of stromal cells,

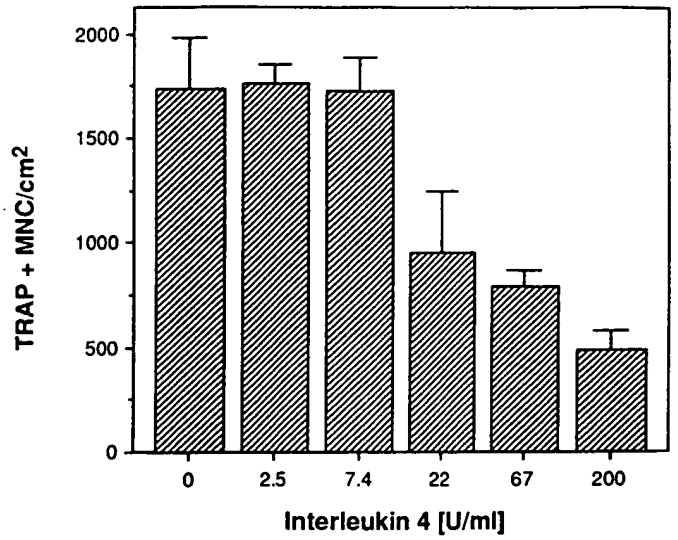

FIG. 3. IL-4 inhibits the osteoclastic cell-forming potential of bone marrow macrophages. Day 4 bone marrow macrophages raised in M-CSF-containing medium were exposed to various concentrations of IL-4 for 48 h before the addition of ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone as described (adherent cell method, Fig. 1C). Osteoclastic cells were counted as described and are presented as the mean ± SEM. Compared to the control, the 22 ($P < 0.0061$), 67 ($P < 0.0016$), and 200 ($P < 0.0001$) U/ml treatment groups were statistically significant.

1,25-$(OH)_2D_3$ (10 nM), and dexamethasone (100 nM). As shown in Fig. 4, IFNγ inhibited osteoclastic cell formation dose dependently, with an ID_{50} between 10–100 U/ml. Maximum inhibition (~85%) occurred with 1000 U/ml IFNγ.

PGE_1, on the other hand, had different effects on osteoclastic cell formation (Fig. 5) when used to treat bone marrow macrophages. Prostanoid exposure actually re-

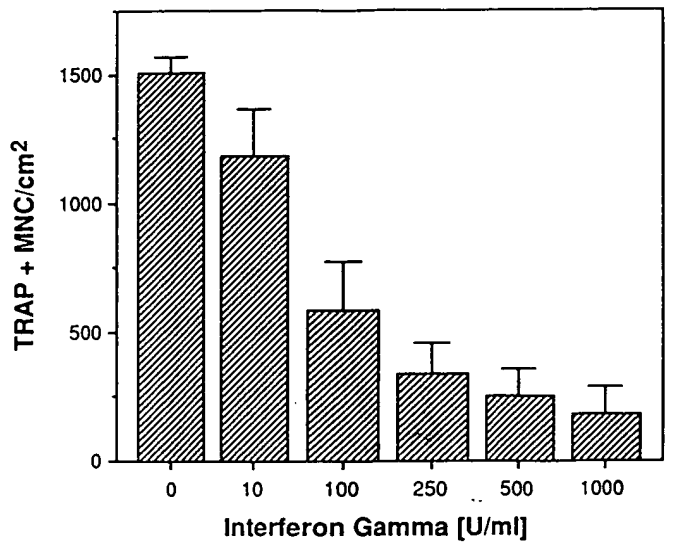

FIG. 4. IFNγ inhibits the osteoclastic cell-forming potential of bone marrow macrophages. Bone marrow macrophages were prepared and treated as described in Fig. 3, except that various amounts of IFNγ were employed instead of IL-4. Osteoclastic cells were counted as described and are presented as the mean ± SEM. All IFNγ treatments at 10 U/ml and above led to significant declines in osteoclastic cell number (10 U/ml, $P < 0.0101$; 100-1000 U/ml, $P < 0.0001$).

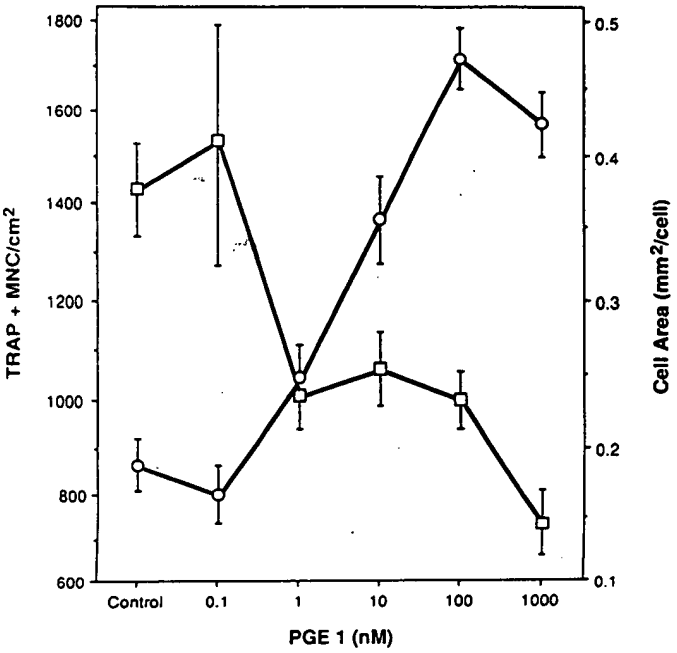

FIG. 5. PGE_1 impacts the osteoclastic cell-forming potential of bone marrow macrophages. Bone marrow macrophages were prepared and treated as described in Fig. 3, except that various amounts of PGE_1 were employed instead of IL-4. Osteoclastic cells were counted (□), and the areas (○) of the largest osteoclastic cells (n = 40/treatment) in each treatment group were measured as described and are presented as the mean ± SEM. Cell area was significantly different from the control value at all PGE_1 levels above 1 nM PGE_1 (1 nM, $P < 0.0745$; 10, 100, and 1000 nM, $P < 0.0001$).

duced osteoclastic cell number in a concentration-dependent manner, with an ED_{50} of about 1 nM. The greatest effect was seen at 1 μM PGE_1, where osteoclastic cell number was 50% of the control value. In contrast to its dampening effect on osteoclastic cell number, however, PGE had a marked impact on the area of the individual osteoclastic cells that developed from the bone marrow macrophages, as the PGE_1-treated cultures contained numerous huge osteoclastic cells. In fact, these large osteoclastic cells became nearly confluent on the plastic surface beneath the stromal cell layer (see Fig. 6). To make a more quantitative assessment of this effect, we used an image analysis method to measure the cell area of the largest osteoclastic cells (40 largest/treatment group) formed in the wells subsequent to PGE_1 exposure. These results are also shown in Fig. 5. As the graph demonstrates, there is an inverse relationship between cell number and size. That is, as the osteoclastic cell number decreased in response to higher PGE_1 levels, the osteoclastic cells that developed became larger in size, with the cross-over point of these parameters being about 1 nM. At 100 nM PGE_1, the cells that developed were 2–3 times larger in area than those in control wells. In accordance with the increased cell area, the larger cells that formed subsequent to PGE_1 exposure contained more nuclei than the controls (data not shown), indicating that an increased degree of cell fusion had occurred. Essentially the same results were obtained for PGE_2 (data not shown).

Akatsu *et al.* (31) previously showed that the effects of PGE on osteoclastic cell formation in a whole bone marrow culture

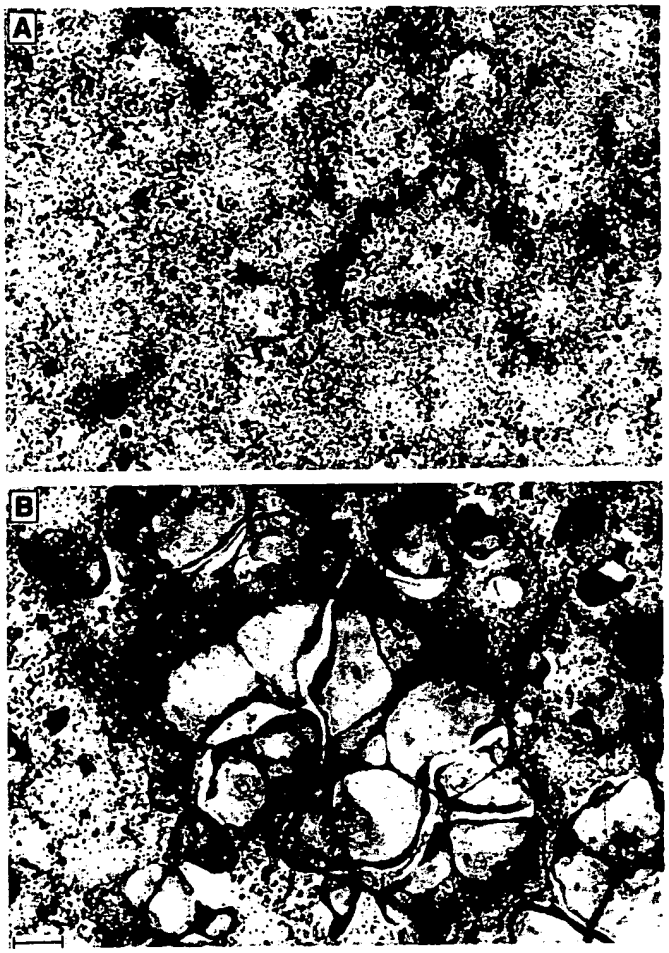

FIG. 6. PGE_1 treatment leads to osteoclastic cells having an increased cell area. Bone marrow macrophages were prepared and treated as described in Fig. 1C with and without 1 μM PGE_1 during the 48-h intermediate period. The cultures were TRAP stained 8 days later, and photomicrographs (×20 magnification) were prepared. A, Control; B, PGE_1. The *calibration bar* represents 250 μm.

model of osteoclastic cell formation could be approximated by use of the cell-permeant cAMP analog, $(Bu)_2$cAMP (31). To determine whether cAMP could play a role in the adherent cell model we developed, we exposed day 4 adherent bone marrow macrophages to various concentrations of either 8-bromo- (8-Br-cAMP) or *N*(6)-benzoyl-cAMP for 48 h, as we had done with the PGEs. After rinsing, ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone were added as outlined (Fig. 1C). Consistent with these previous results, both cAMP analogs (Fig. 7, A and B) had an impact on osteoclastic cell formation, with their effects being qualitatively similar to those observed with PGE_1. At high levels of the cAMP analogs (1 mM), osteoclastic cell number was decreased (~50%), yet cell area was markedly increased (2- to 3-fold), just as had occurred when PGE_1 was employed. The cAMP effect diminished as the concentrations of these agents decreased. At less than 10 μM, the effects of cAMP on osteoclastic cell number and size disappeared.

To exclude the possibility that the cell size-enhancing effect of PGE and cAMP was just an *in vitro* artefact without functional significance, we examined the impact of these

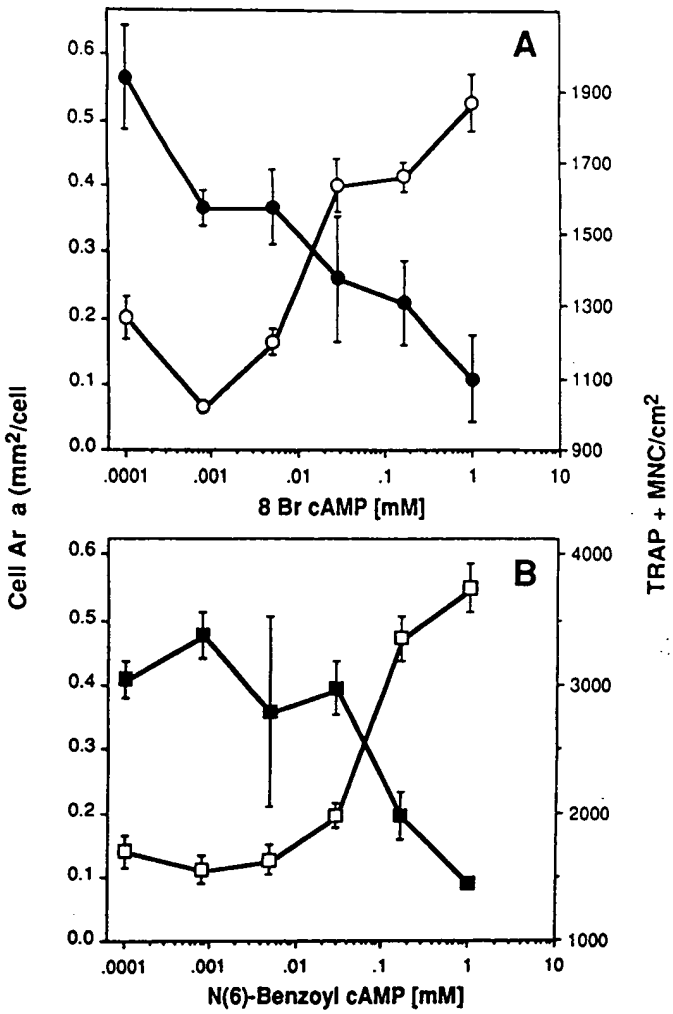

FIG. 7. cAMP derivatives impact the osteoclastic cell-forming potential of bone marrow macrophages. Bone marrow macrophages were prepared and treated as described in Fig. 3, except that various amounts of either 8-Br-cAMP (A) or *N*(6)-benzoyl-cAMP (B) were added instead of IL-4. Osteoclastic cells were counted (● and ■), and the areas (○ and □) of the largest osteoclastic cells (n = 30/treatment) in each treatment group were measured as described and are presented as the mean ± SEM. For cell area for the 8-Br-cAMP group, the treated cells were significantly larger than controls in the 1 mM ($P < 0.0001$), 0.17 mM ($P < 0.0001$), and 0.028 mM ($P < 0.0001$) treatment groups. For cell area for the *N*(6)-benzoyl-cAMP group, the treated cells were significantly larger than controls in the 1 mM ($P < 0.0001$) and 0.167 mM ($P < 0.0001$) treatment groups.

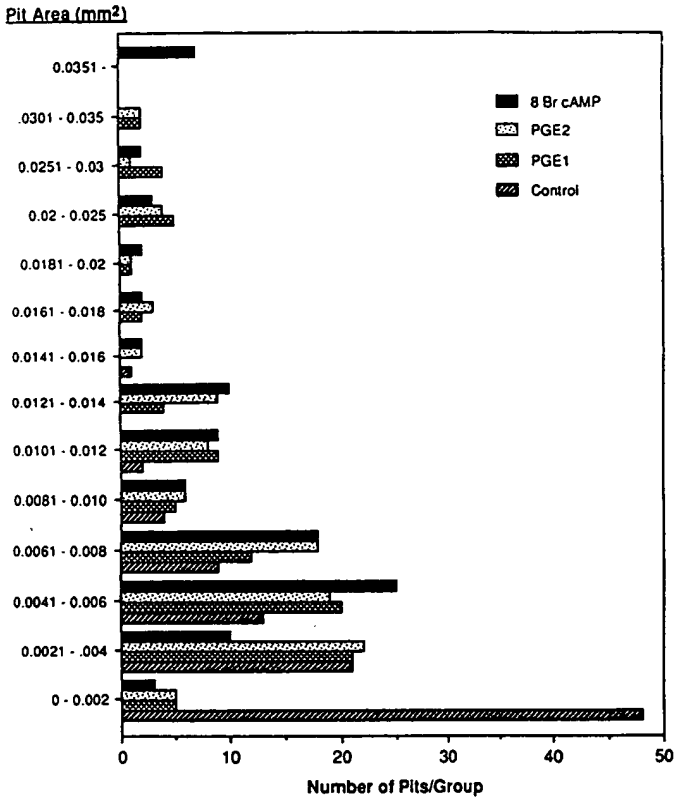

FIG. 8. PGE_1, PGE_2, and 8-Br-cAMP impact resorption lacunae area. Bone marrow macrophages were prepared on slices (n = 3/group) of bovine cortical and treated as described with PGE_1 (1 μM), PGE_2 (1 μM), 8-Br-cAMP (1 mM), or control medium before the addition of ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone as described (Fig. 1C). After an additional 8 days, the slices were processed and stained to demonstrate resorption lacunae (pits). The area of 100 pits from each group was determined by image analysis and plotted in the histogram. The pits in all treatment groups were significantly larger than the control using the Kolmogorov-Smirnov test ($P < 0.0001$).

treatments on resorption lacunae formation. For this experiment, we used the adherent cell method (Fig. 1C), and instead of culturing the cells on plastic, we cultured the bone marrow cells on slices of bovine cortical bone. On day 4, we rinsed nonadherent cells from the wells, and for 2 days the cultures were treated with PGE_1 and PGE_2 (1 μM), 8-Br-cAMP (1 mM), or medium alone. All conditions contained M-CSF (1000 U/ml) to maintain macrophage viability. After this intermediate period, ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone were added for an additional 8-day period. The pit areas were then evaluated by image analysis after the bone slices had been appropriately stained. Figure 8 shows the results of this experiment, in which 100 pits from each treatment condition were measured. Most of the pits that formed under control conditions (~70%) were less than 0.004 mm^2 in area. In contrast, the majority of pits that formed in the PGE (PGE_1, ~75%; PGE_2, ~~75%) and 8-Br-cAMP (~85%) were larger than 0.004 mm^2. No pit in the control group exceeded 0.016 mm^2, whereas pits exceeding this size were found in the groups treated with PGE_1, PGE_2, and 8-Br-cAMP. These data suggest that under the conditions examined, increased osteoclastic cell area on plastic and the capacity for the cells to form pits with an increased area on bone are associated.

In contrast to suppressing the osteoclastic cell-forming potential of bone marrow macrophages, both IL-4 and IFNγ have been shown to augment the capacity of these cells to perform various immune activities (32–35). Interestingly, PGE and cAMP derivatives have been found to antagonize many of the immune-stimulating effects of both of these lymphokines (36–39). To determine whether PGE_1 and 8-Br-cAMP could also antagonize the inhibitory effect of IL-4 on the osteoclastic cell-forming potential of bone marrow macrophages, we added these agents together to the adherent day 4 bone marrow macrophages before the introduction of conditions leading to terminal osteoclastic cell differentia-

tion. As shown in Fig. 9A, PGE_1 partially reversed the inhibitory effects of IL-4 on osteoclastic cell number. Additionally, 8-Br-cAMP also reversed the inhibitory effects of IL-4, similar to what was observed with PGE_1. Importantly, both PGE_1 and 8-Br-cAMP augmented osteoclastic cell area about 4- to 5-fold (Fig. 9B) even when combined with IL-4.

To determine whether the inhibitory effects of IFNγ on osteoclastic cell formation were sensitive to PGE treatment, day 4 bone marrow macrophages were exposed for 48 h to various IFNγ concentrations in the presence and absence of 1 μM PGE_1. Eight days after the introduction of ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone, osteoclastic cell number and size were evaluated. As shown in Fig. 10A, PGE_1 antagonized the inhibitory effects of IFNγ on cell number when

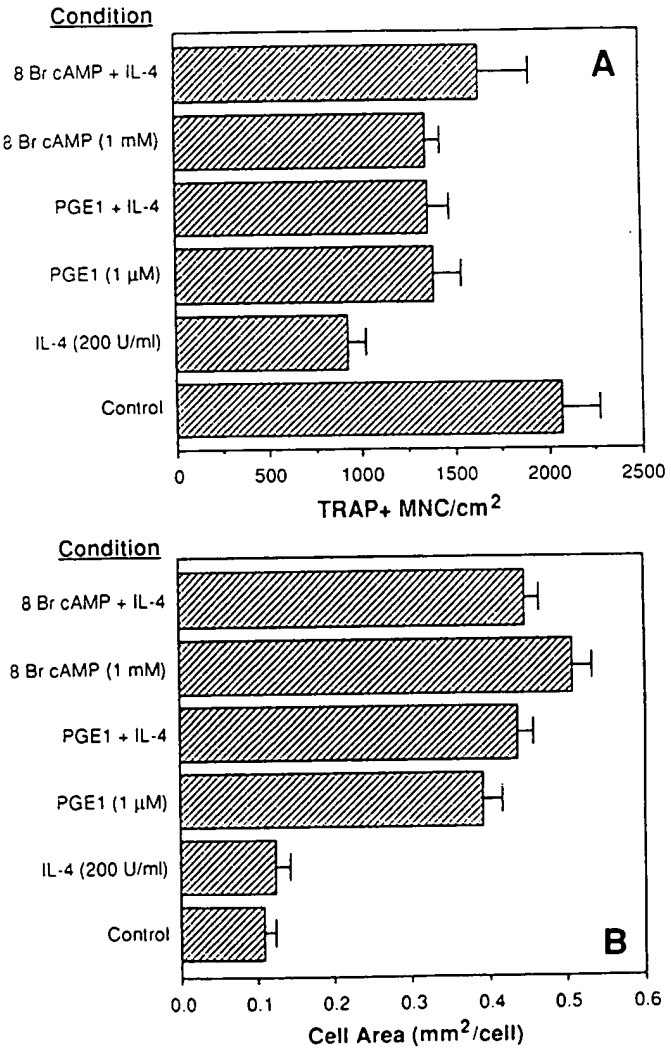

FIG. 9. PGE_1 and 8-Br-cAMP antagonize the effects of IL-4 on osteoclastic cell formation. Bone marrow macrophages were treated with control medium or medium with IL-4, PGE_1, 8-Br-cAMP or various combinations thereof for 48 h before the addition of ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone, as depicted in Fig. 1C. Osteoclastic cell number (A) and area (B) were assessed as described, and the data are presented as the mean ± SEM. For cell area, the P values for the groups were as follows: IL-4 plus PGE_1 *vs.* IL-4, $P < 0.0001$; IL-4 plus 8-Br-cAMP *vs.* IL-4, $P < 0.0001$; PGE_1 *vs.* control, $P < 0.0001$; 8-Br-cAMP *vs.* control, $P < 0.0001$; IL-4 *vs.* control, P = NS.

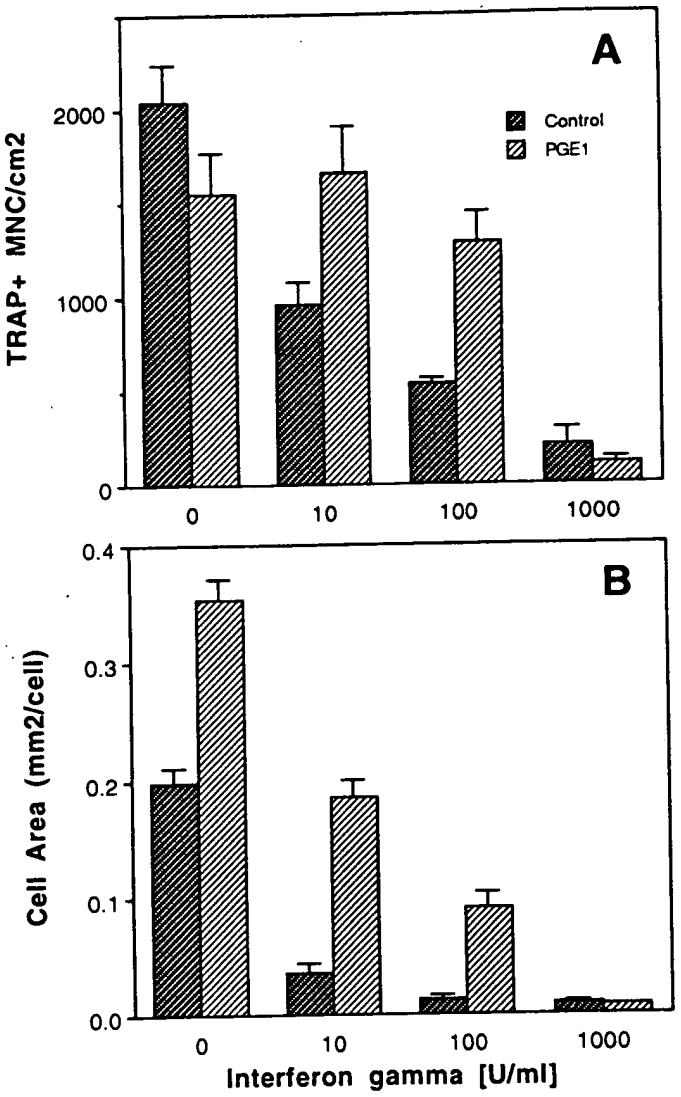

FIG. 10. PGE_1 antagonizes the effects of IFNγ on osteoclastic cell formation. Bone marrow macrophages were treated with control medium or medium with the indicated amounts of IFNγ, PGE_1 (1 μM), or various combinations thereof for 48 h before the addition of ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone, as depicted in Fig. 1C. Osteoclastic cell number (A) and area (B) were assessed as described, and the data are presented as the mean ± SEM. For cell area, the P values for the groups were as follows: 1000 U/ml IFNγ plus PGE_1 *vs.* 1000 U/ml IFNγ, P = NS; 100 U/ml IFNγ plus PGE_1 *vs.* 100 U/ml IFNγ, $P < 0.0001$; 10 U/ml IFNγ plus PGE_1 *vs.* 10 U/ml IFNγ, $P < 0.0001$; PGE_1 *vs.* control, $P < 0.0001$.

this lymphokine was present at 10 and 100 U/ml. PGE_1 treatment also led to the development of larger osteoclastic cells in both control and IFNγ-treated cultures, as demonstrated in Fig. 10B. In contrast, PGE_1 was essentially without effect on cells treated with 1000 U/ml IFNγ, which is a massive level of the factor.

Discussion

In this study, we have shown that the inhibitory effects of IL-4 on osteoclastic cell formation using the continuous coculture model of osteoclastogenesis occurred early in the

culture period. Based on the cross-over experiment, the IL-4 cellular target was present in the bone marrow cells. Further fractionation of the bone marrow cell population, using adherence and M-CSF responsiveness as selection criteria, resulted in a cell population that formed osteoclastic cells after ST2 cell and steroid additions. Using these adherent cells, we observed that IL-4 inhibited osteoclastic cell formation, which is similar to what we had observed when this cytokine was employed in the cross-over experiments or continuous coculture system (11). These data suggest that the IL-4-sensitive target was the bone marrow macrophage based on the cell's adherence and the fact that they were derived from the bone marrow using the monocyte-macrophage lineage-specific growth factor, M-CSF (40). Furthermore, bone marrow cells prepared in this manner have previously been shown to express mannose receptors (22), a phagocytic receptor that is expressed widely in cells of the mononuclear phagocyte system (41).

Surprisingly, treatment of ST2 cells with IL-4 before their combination with bone marrow cells did not lead to impaired osteoclastic cell formation. In hematopoiesis, Peschel *et al.* (6) reported that bone marrow-derived stromal cell monolayers pretreated with IL-4 inhibited hematopoietic colony formation. However, they also demonstrated that glucocorticoids (hydrocortisone) blocked the IL-4-induced inhibitory effect if added during either the IL-4 preincubation phase or the colony formation stage. Because we employed dexamethasone in the coculture medium or added stromal cells after the bone marrow macrophages had already been treated with IL-4 in the adherent cell model, it is unlikely that the production of IL-4-induced inhibitory factors, similar to what Peschel *et al.* (6) observed, could have played a role in our cultures.

Because the monocyte-macrophage presented itself as the likely IL-4 target, we queried whether this cell could be influenced by other agents known to influence *in vitro* osteoclastic cell generation. PGEs and IFNγ are factors that have been shown to stimulate (31) and inhibit (13), respectively, osteoclastic cell formation in murine bone marrow cultures. Consistent with these previous reports, we found that both PGE and IFNγ impacted osteoclastic cell formation when used selectively on bone marrow macrophages before the introduction of ST2 cells, 1,25-$(OH)_2D_3$, and dexamethasone. Surprisingly, the PGEs dose dependently reduced the number of osteoclastic cells. However, prostanoid exposure led to the formation of huge osteoclastic cells that contained increased numbers of nuclei. Because increased cell fusion has led to the presence of the larger osteoclastic cells, it is probable that it has indirectly reduced the number of countable osteoclastic cells. Therefore, we still consider PGEs as stimulants of osteoclastic cell formation, with the most pronounced effect in our system resulting in increased osteoclastic cell size. The capacity for PGE to induce this effect *in vitro* is consistent with the results of Holtrop *et al.* (42), who found osteoclasts formed in PGE-treated fetal rat long bones *in vitro* to be of increased size relative to controls.

In agreement with prior reports, cAMP analogs produced effects similar to those of PGE on both generated osteoclastic cell number and size. Furthermore, it appears that increased osteoclastic cell size on plastic may predict increased resorptive activity, because resorption lacunae that formed on bone slices in PGE- and cAMP-treated cultures were larger in area than those that formed in control cultures. To determine whether the enlarged osteoclastic cells resorbed more bone would require three-dimensional studies of the resorption lacunae that would establish the volume of bone removed. In any event, these findings confirm and extend previous reports examining the effects of PGEs on *in vitro* osteoclastic cell formation (17, 31) by 1) identifying a PGE-sensitive osteoclastic cell progenitor within the bone marrow cell compartment and 2) demonstrating that the same cells targeted by PGE respond to cAMP analogs in a similar manner.

Most importantly, however, PGE_1 antagonized the osteoclastic cell-inhibiting effects of IL-4 and IFNγ. Although these observations suggested that IL-4- and IFNγ-mediated suppression of osteoclastic cell formation could have been mediated by impaired PGE production in our cultures, the failure of indomethacin (1 and 10 μM) to produce this inhibitory effect (data not shown) when used during the same 48-h treatment period essentially discounts this possibility.

The agents we tested have effects on osteoclastic cell proliferation that appear to be dissociated from their impact on M-CSF-driven bone marrow macrophage proliferation. For instance, IFNγ (43) and PGE (44) are both known to inhibit macrophage proliferation, yet these agents have opposing effects on the osteoclastic cell-forming potential of the same cells. Along these same lines, the fact that IL-4 can enhance macrophage proliferation in the presence of M-CSF is also known (45), and we confirmed this observation (data not shown). Therefore, we conclude that the effects of IL-4, PGE, IFNγ, and the cAMP analogs on osteoclastic cell formation are not a direct reflection of their impact on M-CSF-driven bone marrow macrophage proliferation.

Despite the fact that our studies have led to the identification of macrophages as the target cell in our system, the mechanism(s) driven by IL-4, IFNγ, and PGE remains undefined. A provocative hypothesis that ties together many of our observations can be proposed based on a recent report describing the osteopetrosis that develops in c-*fos* knockout mice (46). In this study, the researchers present convincing evidence that osteoclast differentiation from macrophages is blocked in c-*fos*-deficient animals, indicating that c-*fos* induction in osteoclast precursors may be pivotal for terminal differentiation. That this block may occur at the level of the macrophage is supported by the presence of numerous macrophages in the bone marrow of the c-*fos*-deficient osteopetrotic animals. These results raise the obvious possibility that alterations in macrophage c-*fos* induction could explain the findings observed in our study. In support of this contention, both IL-4 and IFNγ have been shown to suppress c-*fos* induction in monocyte/macrophages (47, 48), and in our system, both agents inhibit osteoclastic cell formation from bone marrow macrophages. Furthermore, agents that elevate or mimic cAMP in macrophages, such as PGE and 8-Br-cAMP, have been shown to cause prolonged elevations of macrophage c-*fos* mRNA levels (48, 49), and in our model they stimulate osteoclastic cell development. These data raise the possibility that inflammatory cytokines and lipids may influence osteoclast development in part through their ef-

fects on signaling pathways that alter c-*fos* expression in macrophages.

Based on our results, we formulated a variation of a model (see Fig. 11) of *in vitro* osteoclastic cell formation originally presented by Suda *et al.* (18). In our adapted model, the monocyte-macrophage is a candidate osteoclastic cell progenitor subject to local conditions, including the presence of immune and inflammatory mediators. In constructing this model, we took into consideration previous studies of lymphokine-mediated macrophage activation. Previous investigators have established that both IFNγ and IL-4 have immunomodulatory effects on macrophages, including increases in both class II antigen expression (32) and, in the case of IL-4, induction of multinucleation (12, 50). Furthermore, PGEs and cAMP analogs antagonize the effects of both lymphokines on macrophages (38, 51) while, based on our experiments, priming the same cells to differentiate into osteoclastic cells, provided that local conditions are met. Therefore, we postulate that the relative actions of the lymphokines and PGs on the macrophage determine the cell's ultimate capacity to differentiate into an osteoclastic cell. Interestingly, the effects of these agents appear to occur during an intermediate period of differentiation before the introduction of 1,25-$(OH)_2D_3$, dexamethasone, and stromal cells. These latter factors and cells apparently need to be present during a proposed terminal differentiation period that concludes with the appearance of osteoclastic cells.

With regard to further studies, the adherent cell method provides a model to test other potential osteoclastogenesis-influencing agents in a defined (albeit artefactual) manner. There are four different identifiable periods in this method that could be targeted, as shown in Figs. 1 and 11, including early (days 0–1), growth (days 2–4), intermediate differentiation (days 5–6), and terminal differentiation (days 7–14) periods. Our initial results with IL-4 focussed our attention at the intermediate differentiation period, where we proceeded to test other osteoclastogenesis-modifying factors. Although our findings identify a narrow window of time (days 5 and 6, intermediate differentiation period) as a factor-sensitive interval, we did not examine the possibility that the same agents explored above could have effects if used during any of the other intervals.

In the case of IL-4, Riancho *et al.* (12) showed that IL-4's inhibition of osteoclastic cell formation was mediated by the cytokine's effects on their culture before the appearance of osteoclastic cells. They could only theorize that IL-4 acted on putative uncommitted osteoclastic cell precursors in the bone marrow or spleen because they had employed mixed cell populations in their cultures. In contrast to speculation, we identified the IL-4 cellular target in the marrow compartment and showed that the same cell is responsive to other treatments.

In summary, our results identify the bone marrow macrophage as a mononuclear phagocyte with the potential to function as an efficient osteoclastic cell precursor. The degree to which the cell is actually committed to the osteoclast pathway appears to be influenced by soluble mediators produced by cells of the immune system. Because IL-4 and IFNγ appear to direct macrophage differentiation toward immune activities and away from osteoclastic cell formation, there may be a common specific step in the mechanisms driven by these factors, sensitive to PGEs and potentially other cAMP-generating conditions, that is a pivotal determinant of macrophage differentiation. The elucidation of this putative maturation "checkpoint" could potentially provide the basis for antiresorptive therapies.

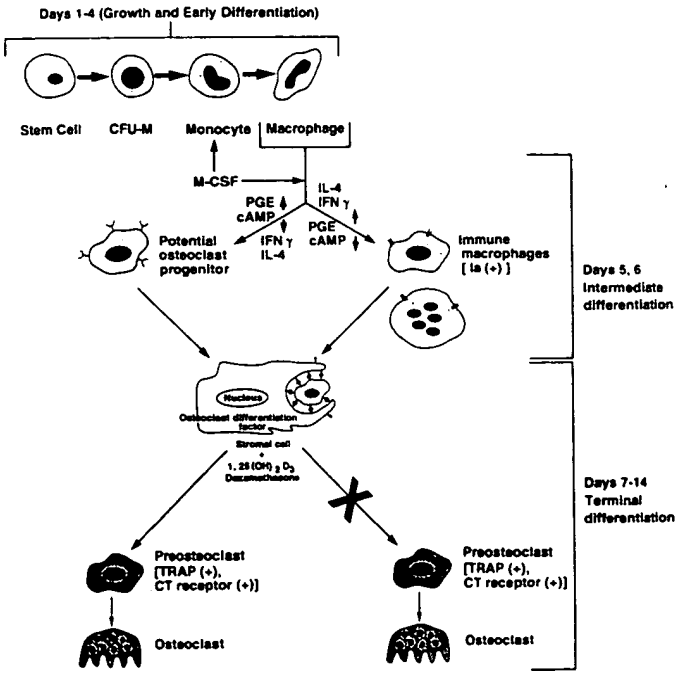

FIG. 11. Model of osteoclastic cell formation from bone marrow macrophages [adapted from Suda *et al.* (18)]. Ia, Class II antigen; CT, calcitonin.

References

1. **Howard M, John F, Hilfiker M, Johnson B, Takatsu K, Kamaoka T, Paul WE** 1982 Identification of a T cell-derived B cell growth factor distinct from interleukin 2. J Exp Med 155:914–923
2. **Kupper T, Horowitz M, Lee F, Robb R, Flood PM** 1987 Autocrine growth of T cells independent of interleukin 2: identification of interleukin 4 (IL-4, BSF-1) as an autocrine growth factor for a cloned antigen-specific helper T cell. J Immunol 138:4280–4287
3. **Mosmann TR, Bond MW, Coffman RL, Ohara J, Paul WE** 1986 T-Cell and mast cell lines respond to B-cell stimulatory factor 1. Proc Natl Acad Sci USA 83:5654
4. **Abramson S, Gallin JI** 1990 IL-4 Inhibits superoxide production by human mononuclear phagocytes. J Immunol 144:625–630
5. **Peschel C, Paul WE, Ohara J, Green I** 1987 Effects of B cell stimulatory factor-1/interleukin 4 on hematopoietic progenitor cells. Blood 70:254–263
6. **Peschel C, Green I, Paul WE** 1989 Interleukin-4 induces a substance in bone marrow stromal cells that reversibly inhibits factor-dependent and factor-independent cell proliferation. Blood 73:1130–1141
7. **Rennick D, Yang G, Muller SC, Smith C, Arai N, Takabe Y, Gemmell L** 1987 Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. Proc Natl Acad Sci USA 84:6889–6893
8. **Watanabe K, Tanaka Y, Morimoto I, Eto S** 1990 Interleukin 4 as a potent inhibitor of bone resorption. Biochem Biophys Res Commun 172:1035–1041
9. **Mundy GR, Ibbotson KJ, D'Souza SM, Simpson EL, Jacobs JW, Martin TJ** 1984 The hypercalcemia of malignancy, clinical implications and pathogenetic mechanisms. N Engl J Med 310:1718–1727
10. **Nakano Y, Watanabe K, Morimoto I, Okada Y, Ura K, Sato K,**

Kasono K, Nakamura T, Eto S 1994 Interleukin-4 inhibits spontaneous and parathyroid hormone-related protein-stimulated osteoclast formation in mice. J Bone Miner Res 9:1533–1539

11. **Shioi A, Teitelbaum SL, Ross FP, Suzuki H, Ohara J, Lacey DL** 1991 Interleukin 4 inhibits murine osteoclast formation *in vitro*. J Cell Biochem 47:272–277
12. **Riancho JA, Zarrabeitia MT, Mundy GR, Yoneda T, Gonzalez-Macias J** 1993 Effects of interleukin-4 on the formation of macrophages and osteoclast-like cells. J Bone Miner Res 8:1337–1344
13. **Kasono K, Sato K, Sato Y, Tsushima T, Shizume K, Demura H** 1993 Inhibitory effect of interleukin-4 on osteoclast-like cell formation in mouse bone marrow culture. Bone Miner 21:179–188
14. **Gowen M, Nedwin GE, Mundy GR** 1986 Preferential inhibition of cytokine-stimulated bone resorption by recombinant interferon gamma. J Bone Miner Res 1:469–474
15. **Gowen M, Mundy GR** 1986 Actions of recombinant interleukin 1, interleukin 2, and interferon-γ on bone resorption *in vitro*. J Immunol 136:2478–2482
16. **Dietrich JW, Goodson JM, Raisz LG** 1975 Stimulation of bone resorption by various prostaglandins in organ culture. Prostaglandins 10:231–240
17. **Collins DA, Chambers TJ** 1992 Prostaglandin E2 promotes osteoclast formation in murine hematopoietic cultures through an action on hematopoietic cells. J Bone Miner Res 7:555–561
18. **Suda T, Takahashi N, Martin TJ** 1992 Modulation of osteoclast differentiation. Endocr Rev 13:66–80
19. **Ohara J, Colian JE, Zoon K, Maloy WL, Paul WE** 1987 High-efficiency purification and chemical characterization of B cell stimulatory factor-1/interleukin 4. J Immunol 139:1127–1134
20. **Ohara J,** 1989 Interleukin-4: Molecular structure and biochemical characteristics, biological function, and receptor expression. In: Cruse JM, Lewis REJ (eds) The Year in Immunology: Immunoregulatory Cytokines and Cell Growth. Karger, Basel, pp 126–159
21. **Lacey DL, Chappel JC, Teitelbaum SL** 1987 Interleukin 1 stimulates proliferation of a non-transformed T lymphocyte line in the absence of a co-mitogen. J Immunol 139:2649–2655
22. **Clohisy DR, Bar-shavit Z, Chappel JC, Teitelbaum SL** 1987 1,25-Dihydroxyvitamin D3 modulates bone marrow macrophage precursor proliferation and differentiation. J Biol Chem 262:15922–15929
23. **Stanley ER, Heard PM** 1977 Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J Biol Chem 252:4305–4312
24. **Perkins SL, Teitelbaum SL** 1991 1,25-Dihydroxyvitamin D_3 modulates colony-stimulating factor 1 receptor binding by murine bone marrow macrophage precursors. Endocrinology 128:303–311
25. **Arnett TR, Dempster DW** 1987 A comparative study of disaggregated chick and rat osteoclasts *in vitro*: effects of calcitonin and prostaglandins. Endocrinology 120:602–608
26. **Lacey DL, Erdmann JM, Tan HL, Ohara J** 1993 Murine osteoblast interleukin 4 receptor expression: upregulation by 1,25 dihydroxyvitamin D3. J Cell Biochem 53:122–134
27. **Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T** 1990 Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proc Natl Acad Sci USA 87:7260–7264
28. **Wiktor-Jedrezejczak W, Bartocci A, Ferrante AW, Ahmed-Ansari A, Sell KW, Pollard JW, Stanley ER** 1990 Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (*op/op*) mouse. Proc Natl Acad Sci USA 87:4828–4832
29. **Yoshida H, Hayashi S-I, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S-I** 1990 The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442–444
30. **Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER** 1982 Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell 28:71–81
31. **Akatsu T, Takahashi N, Debari K, Morita I, Murota S, Nagata N, Takatani O, Suda T** 1989 Prostaglandins promote osteoclastlike cell formation by a mechanism involving cyclic adenosine 3′,5′-monophosphate in mouse bone marrow cell cultures. J Bone Miner Res 4:29–35
32. **Cao H, Wolff RG, Meltzer MS, Crawford RM** 1989 Differential regulation of class II MHC determinants on macrophages by IFN-γ and IL-4. J Immunol 143:3524–3531
33. **Crawford RM, Finbloom DS, Ohara J, Paul WE, Meltzer MS** 1987 B Cell stimulatory factor-1 (interleukin 4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J Immunol 139:135–141
34. **Stuart PM, Zlotnik A, Woodward JG** 1988 Induction of class I and class II MHC antigen expression on murine bone marrow-derived macrophages by IL-4 (B cell stimulatory factor 1). J Immunol 140: 1542–1547
35. **Littman BH, Dastvan F, Carlson PL, Sanders KM** 1989 Regulation of monocyte/macrophage C2 production and HLA-DR expression by IL-4 (BSF-1) and IFN-γ. J Immunol 142:520–525
36. **Zimmer T, Jones PP** 1990 Combined effects of tumor necrosis factor-α, prostaglandin E2, and corticosterone on induced Ia expression on murine macrophages. J Immunol 145:1167–1175
37. **Schultz R, Pavlidis N, Stylos W, Chirigos M** 1978 Regulation of macrophage tumoricidal function: a role for prostaglandins of the E series. Science 202:320–321
38. **Snyder DS, Beller DI, Unanue ER** 1982 Prostaglandins modulate macrophage Ia expression. Nature 299:163–165
39. **Ivashkiv LB, Glimche LH** 1991 Repression of class II major histocompatibility genes by cyclic AMP is mediated by conserved promoter elements. J Exp Med 174:1583–1592
40. **Stanley ER,** 1992 The biology and action of colony stimulating factor-1. In: van Furth R (ed) Mononuclear Phagocytes. Biology of Monocytes and Macrophages. Kluwer, Boston, pp 13–20
41. **Ezekowitz RA, Stahl PD** 1988 The structure and function of vertebrate mannose lectin-like proteins. J Cell Sci [Suppl] 9:121–133
42. **Holtrop ME, Raisz LG, King GJ** 1978 The response of osteoclasts to prostaglandin and osteoclast activating factor as measured by ultrastructural morphometry. In: Horton JE, Tarpley TM, Davis WF (eds) Mechanisms of Localized Bone Loss. Information Retrieval, Washington DC, pp 13
43. **Vairo G, Royston AK, Hamilton JA** 1992 Biochemical events accompanying macrophage activation and the inhibition of colony-stimulating factor-1-induced macrophage proliferation by tumor necrosis factor-alpha, interferon-gamma, and lipopolysaccharide. J Cell Physiol 151:630–641
44. **Moore RN, Pitruzzello FJ, Larsen HS, Rouse BT** 1984 Feedback regulation of colony-stimulating factor (CSF-1)-induced macrophage proliferation by endogenous E prostaglandins and interferon-alpha/beta. J Immunol 133:541–543
45. **Chen BD-M, Sensenbrenner L, Fan K, Run Q** 1992 Murine recombinant IL-4 is a bifunctional regulator of macrophage growth induced by colony-stimulating factors. J Immunol 148:753–759
46. **Grigoriadis AE, Wang Z-Q, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF** 1994 c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science 266:443–448
47. **Dokter WHA, Esselink MT, Halie MR, Vellenga E** 1993 Interleukin-4 inhibits the lipopolysaccharide-induced expression of c-jun and c-fos messenger RNA and activator protein-1 binding activity in human monocytes. Blood 81:337–343
48. **Radzioch D, Varesio L** 1991 c-Fos mRNA expression in macrophages is downregulated by interferon-γ at the posttranscriptional level. Mol Cell Biol 11:2718–2722
49. **Bravo R, Neuberg M, Burckhardt J, Almendral J, Wallich R, Muller R** 1987 Involvement of common and cell type-specific pathwaly in cfos gene control: stable induction by cAMP in macrophages. Cell 48:251–260
50. **McInnes A, Rennick DM** 1988 Interleukin 4 induces cultured monocytes/macrophages to form giant multinucleated cells. J Exp Med 167:598–611
51. **Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA** 1989 Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tyumor necrosis factor α, interleukin 1, and prostaglandin E_2. Proc Natl Acad Sci USA 86:3803–3807